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Effects of high-flux hemodialysis on oxidant stress

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Background. Neutrophil oxygen radical production is increased in end-stage renal disease (ESRD) patients and it is further enhanced during dialysis with low-flux cellulosic membranes. This increased oxygen radical production may contribute to the protein and lipid oxidation observed in ESRD patients. We tested the hypothesis that high-flux hemodialysis does not increase oxygen radical production and that it is not associated with protein oxidation.

Methods. Neutrophil oxygen radical production was measured during dialysis with high-flux dialyzers containing polysulfone and cellulose triacetate membranes. Free sulfhydryl and carbonyl groups and advanced oxidation protein products were measured to assess plasma protein oxidation.

Results. Pre-dialysis, neutrophil oxygen radical production was significantly greater than normal and increased significantly as blood passed through the dialyzer in the first 30 minutes of dialysis. Post-dialysis, however, neutrophil oxygen radical production had decreased and was not different from normal. Pre-dialysis, significant plasma protein oxidation was evident from reduced free sulfhydryl groups, increased carbonyl groups, and increased advanced oxidation protein products. Post-dialysis, plasma protein free sulfhydryl groups had increased to normal levels, while plasma protein carbonyl groups increased slightly, and advanced oxidation protein products remained unchanged.

Conclusions. The results of this study show that neutrophil oxygen radical production normalizes during high-flux dialysis, despite a transient increase early in dialysis. This decrease in oxygen radical production is associated with an improvement in some, but not all, measures of protein oxidation.

Oxygen radicals are implicated as mediators of renal injury in diverse experimental models of renal disease [1] and of accelerated atherosclerosis in hemodialysis patients through the oxidation of low-density lipoproteins [2, 3]. It has also been suggested that oxidant exposure may contribute to the high rate of cardiovascular disease in these patients [4]. A number of studies have demonstrated oxidation of both proteins and lipids in hemodialysis patients. Increased plasma protein oxidation is evidenced by changes in the concentration of free sulfhydryl groups, carbonyl groups, and 3-chlorotyrosine on plasma proteins [5–8], and the appearance of advanced oxidation protein products (AOPP) [9]. Studies of lipid peroxidation have produced ambiguous results, with levels of lipid peroxidation products being reported as normal [10, 11] or increased [12, 13]. Similarly, in vitro measurements of the lag time for copper-induced low-density lipoprotein (LDL) oxidation have yielded values less than [3] and not different from [14, 15] normal. The discrepancies between these studies may reflect the short plasma half-lives of lipid peroxidation products and the relative non-specificity of the tests [16]. More recently, a sensitive and specific gas chromatography-mass spectrometry assay was used to measure plasma concentrations of esterified F2-isoprostanes, which are chemically stable products of arachidonic acid oxidation [17]. This study found significantly higher levels of esterified F2-isoprostanes in hemodialysis patients than in normal subjects. Taken together, these studies support the hypothesis that end-stage renal disease (ESRD) is associated with both protein and lipid oxidation.

We have demonstrated that neutrophils of hemodialysis patients are primed for an enhanced respiratory burst following stimulation with *Staphylococcus aureus* [18]. This increase in respiratory burst activity may contribute to protein and lipid oxidation in hemodialysis patients. The protein and lipid oxidation might be exacerbated if the dialyzer membrane acted as a further stimulus to oxygen radical production. We have previously shown that exposure to low-flux cellulosic dialysis membranes is sufficient to both further prime and stimulate oxygen radical release by neutrophils [19]. However, there is little information on the impact of other membranes on oxygen radical production. The purpose of this study was to determine the impact of hemodialysis with high-flux polysulfone and cellulose triacetate membranes on neutrophil respiratory burst activity and protein oxidation.

METHODS

Subjects

Twelve chronic hemodialysis patients (four women and eight men) were enrolled in the study. Inclusion criteria

Key words: High-flux hemodialysis, neutrophil, oxygen radicals, oxidant stress, protein oxidation.

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were a stable hemodialysis prescription, a well-functioning native fistula or Gore-Tex graft (W.L. Gore, Flagstaff, AZ, USA), and an absence of clinical evidence of infection during the month prior to the study. One patient received a renal transplant before completing the protocol; the data from this patient were omitted from this report. The average age of the remaining 11 patients was 51 ± 5 years and their average time on dialysis was 49 ± 11 months. The cause of ESRD was diabetes in five patients, glomerulonephritis in three patients, hypertension in two patients, and was unknown in one patient.

Blood samples for determining normal values of neutrophil respiratory burst activity and markers of protein oxidation were obtained from healthy volunteers, both men and women, who ranged in age from 23 to 54 years. At the time blood samples were obtained, all control subjects had been free of infection for at least 2 weeks and were taking no medications, including no anti-inflammatory drugs in the previous 48 hours.

The Human Studies Committee of the University of Louisville approved the study and informed consent was obtained from each subject before each was enrolled in the study.

Study design

Each patient underwent a single treatment with a dialyzer containing polysulfone membrane (F80A, Fresenius USA, Lexington, MA, USA) and a single treatment with a dialyzer containing cellulose triacetate membrane (CT190G, Baxter Healthcare Corp., Deerfield, IL, USA). The two dialyzers were used in random order and a new dialyzer was used for each treatment. All treatments were performed using a volume control dialysate delivery system (Model 550, Baxter Healthcare Corp.).

All treatments were performed according to the patients' routine prescriptions, except that the blood flow rate was set at 300 mL/min. The treatment time was 3.7 ± 0.2 hours and the dialysate flow rate was 700 mL/min. Anticoagulation was achieved by a loading dose (2523 ± 296 IU) and constant infusion (1818 ± 155 IU/hour) of heparin. The patients' pre-dialysis weight was 81.1 ± 5.2 kg and fluid removal during dialysis was 3.4 ± 0.2 kg. None of these treatment parameters differed significantly between the two dialyzers.

Neutrophil respiratory burst activity and markers of protein oxidation were determined before, during, and after dialysis with each dialyzer. A pre-dialysis blood sample was obtained immediately following insertion of the access needles and withdrawal of 3 mL blood to clear any plasma or tissue components activated during needle insertion. Subsequent blood samples were obtained from the arterial and venous lines of the dialyzer after 1, 3, 5, 15, and 30 minutes of dialysis. A post-dialysis sample was obtained from the arterial line at the end of dialysis. To standardize the timing of sample collection, the saline prime was not discarded at the initiation of dialysis. Instead, the blood circuit was completed, the blood flow rate immediately set to 300 mL/min, and zero time taken as the moment when blood first entered the dialyzer. Neutrophil counts and respiratory burst activity were determined in all samples. Markers of protein oxidation were determined in pre- and post-dialysis samples.

Neutrophil count

Blood for white cell count and differential was collected in ethylenediaminetetraacetic acid (EDTA) and analyzed by routine clinical laboratory methods.

Neutrophil respiratory burst activity

Blood for determination of resting and stimulated respiratory burst activity was collected in acid citrate dextrose (ACD) (National Institutes of Health formula A). Respiratory burst activity was determined by the measurement of H_2O_2 production using a flow cytometric method as previously described [20]. Samples were analyzed for phagocytosis and stimulated H_2O_2 production by flow cytometry (Epics Profile II, Coulter, Hialeah, FL, USA). Data are presented as the mean channel of fluorescence intensity. The flow cytometer was calibrated before the analysis of each set of samples with Standard-Brite beads (Coulter).

Markers of plasma protein oxidation

Blood for determination of markers of plasma protein oxidation was collected in EDTA. The plasma was separated immediately by centrifugation and stored at −70°C until analysis. Measurements of free sulfhydryl groups and carbonyl groups on plasma protein and AOPP were used to assess the extent of plasma protein oxidation. Plasma protein free sulfhydryl groups were determined by the method of Ellman, as described by Sedlak and Lindsay [21], and as modified by Hu et al [22]. Briefly, 50 μL of plasma was added to disposable cuvettes containing 1 mL 0.1 mol/L Tris (pH 8.2) containing 10 mmol/L EDTA and 50 μL of 10 mmol/L 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in methanol. A blank, prepared in the same way but without DTNB, was run with each sample and all samples were assayed in triplicate. Following incubation for 15 minutes at room temperature, the absorbance was read at 412 nm on a spectrophotometer. After subtraction of reagent and sample blanks, the concentration of free sulfhydryl groups was determined using the 2-nitro-5-thiobenzoic acid (TNB) extinction coefficient of 14,100 $M^{-1}\,cm^{-1}$ [23]. Plasma protein carbonyl groups were determined by the method of Buss et al [24] as modified by Winterbourn and Buss [25], using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Zentech Protein Carbonyl Kit, Zenith Technology Corp., Dunedin, New Zealand). All samples were assayed in triplicate. AOPP were deter-

Table 1. Neutrophil counts during high-flux hemodialysis

Treatment time *minutes*	Neutrophil count $10^3/\mu L$	
	F80A	CT190G
Pre-dialysis	3.75 ± 0.43	3.80 ± 0.37
1	3.71 ± 0.46	3.67 ± 0.47
3	3.59 ± 0.49	3.37 ± 0.62
5	3.31 ± 0.50	2.97 ± 0.63
15	3.27 ± 0.47	3.27 ± 0.54
30	3.56 ± 0.49	3.89 ± 0.50
Post-dialysis	3.59 ± 0.38	3.67 ± 0.33

Data are presented as mean ± SEM for $N = 11$ for each dialyzer. Neutrophil counts were corrected for hemoconcentration using concurrent hematocrit values. Results of the statistical analysis are presented in the text.

mined using the method of Witko-Sarsat et al [9]. Briefly, samples were diluted 1:5 in buffer and 200 μL added to the wells of a microtiter plate. Acetic acid (20 μL) was added to each well and the plate incubated at room temperature for 2 minutes on an agitator. The absorbance was then read at 340 nm using a microplate reader. AOPP concentrations were calculated in terms of chloramine-T equivalents using a calibration curve constructed from standards prepared by the addition of 10 μL 1.16 mol/L potassium iodide to varying concentrations of chloromine-T. Plasma protein concentrations were measured by the biuret method (Sigma Chemical Co., St. Louis, MO, USA).

Data analysis

Neutrophil counts were corrected for hemoconcentration arising from ultrafiltration using concurrent hematocrit values. The effects of dialyzer type and time on each parameter were assessed by a two-way analysis of variance for repeated measures. Values obtained at the outlet of the dialyzer were compared to those obtained at the inlet using Student *t* test for paired data. A *P* value less than 0.05 was considered significant. Data are presented as mean ± SEM.

RESULTS

Neutrophil count

Changes in neutrophil count with time are presented in Table 1. The neutrophil count decreased significantly during the first 15 minutes of dialysis ($P = 0.001$), both in terms of the actual cell count and following correction for hemoconcentration. By 30 minutes the neutrophil count was not different from the pre-dialysis value. Changes in neutrophil count did not differ between the two dialyzers ($P = 0.174$).

Neutrophil respiratory burst activity

There was no difference between the two dialyzers in their effect on respiratory burst activity ($P = 0.270$ and $P = 0.170$ for resting and stimulated H_2O_2 production, respectively). Resting and *S. aureus*-stimulated H_2O_2 production was significantly increased above normal at the beginning of dialysis (Figs. 1 and 2). There was no change in respiratory burst activity in neutrophils entering the dialyzer during the first 30 minutes of dialysis; however, by the end of dialysis both resting and stimulated H_2O_2 production had decreased significantly ($P < 0.001$) to values within the normal range. The change in stimulated H_2O_2 production was not due to a change in the level of phagocytosis, which did not change from pre- to post-dialysis (data not shown). While respiratory burst activity decreased from pre- to post-dialysis, passage of blood through the dialyzer was associated with a significant increase in resting and stimulated H_2O_2 production after 15 minutes of dialysis.

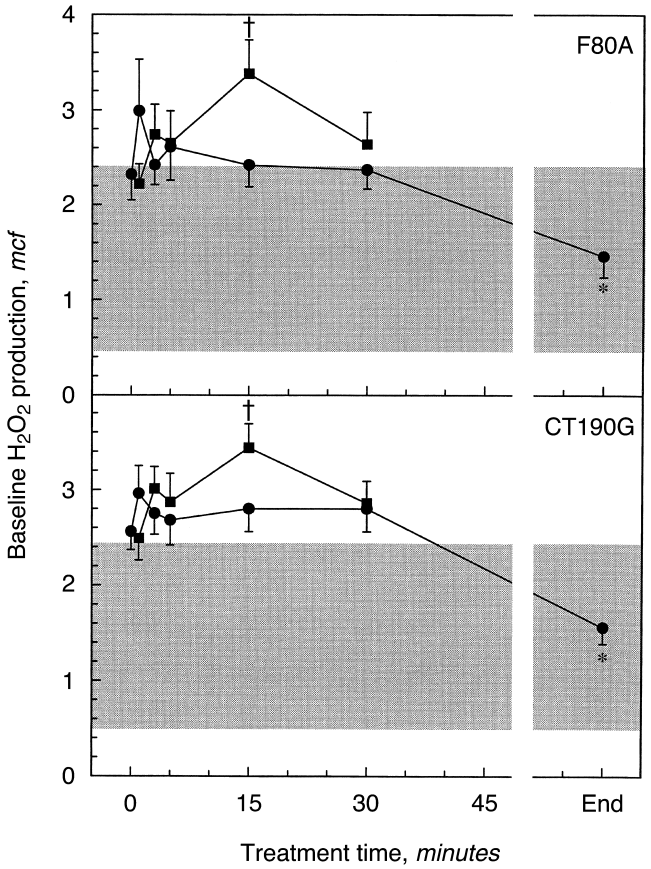

Fig. 1. Resting neutrophil respiratory burst activity during hemodialysis with F80A and CT190G dialyzers. Data are presented as mean ± SEM for $N = 11$. Measurements made at the inlet to the dialyzer are indicated by (●) and measurements made at the outlet to the dialyzer are indicated by (■). *, Significantly different from other time points ($P < 0.001$); †, Significantly different from arterial ($P < 0.001$). The shaded area represents the range for normal subjects (mean ± 2 SD, $N = 18$).

Plasma protein oxidation

Concentrations of markers of protein oxidation are presented in Table 2. There was no difference between

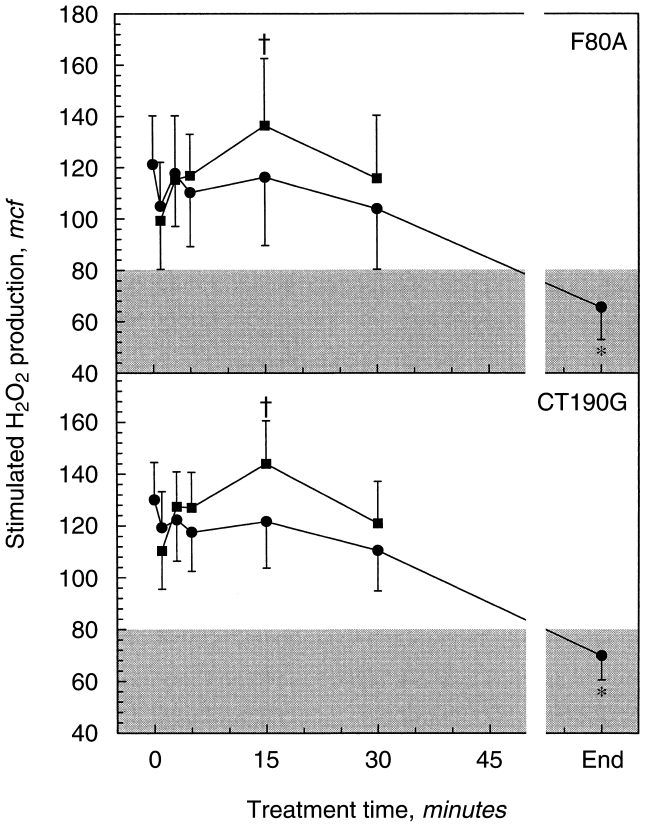

Fig. 2. ***Staphylococcus aureus*–stimulated neutrophil respiratory burst activity during hemodialysis with F80A and CT190G dialyzers.** Data are presented as mean ± SEM for $N = 11$. Measurements made at the inlet to the dialyzer are indicated by (●) and measurements made at the outlet to the dialyzer are indicated by (■). *, Significantly different from other time points ($P < 0.002$); †, Significantly different from arterial ($P < 0.05$). The shaded area represents the range for normal subjects (mean ± 2 SD, $N = 18$).

dialyzers in the concentrations of markers of protein oxidation pre- and post-dialysis ($P = 0.698$ for plasma protein free sulfhydryl groups, $P = 0.963$ for protein carbonyls, and $P = 0.715$ for AOPP). Pre-dialysis, the concentration of plasma protein free sulfhydryl groups was significantly less than normal ($P < 0.001$), while concentrations of plasma protein carbonyl groups ($P = 0.007$) and AOPP ($P < 0.001$) were significantly greater than normal. Dialysis was associated with an increase in the concentration of plasma protein free sulfhydryl groups ($P < 0.001$), and post-dialysis the concentration did not differ from normal ($P = 0.817$). Similar results were obtained when plasma protein free sulfhydryl groups were expressed as nanomole per milligram of protein, calculated using total plasma protein concentrations (data not shown). In contrast to the normalization in concentration observed for plasma protein free sulfhydryl groups, the concentration of plasma protein carbonyl groups increased slightly, but significantly ($P = 0.029$) over the course of dialysis and remained significantly greater than normal post-dialysis ($P < 0.001$). Dialysis had no effect on the concentration of AOPP ($P = 0.321$) and concentrations of AOPP remained significantly greater than normal post-dialysis ($P = 0.001$).

DISCUSSION

The results of this study demonstrate that hemodialysis patients are in a state of oxidant stress. Pre-dialysis, resting and stimulated neutrophil oxygen radical production is significantly increased above normal and there is significant protein oxidation, as demonstrated by a reduction in plasma protein free sulfhydryl groups and increases in plasma protein carbonyl groups and advanced oxidation protein products.

Dialysis with high-flux membranes fabricated from either polysulfone or cellulose triacetate transiently activated neutrophils early in the course of hemodialysis, as evidenced by neutropenia and an increase in respiratory burst activity as blood transited the dialyzer. The complement fragment, C5a, has been reported to stimulate [26] and, at substimulatory concentrations, to prime [27] respiratory burst activity in neutrophils. Thus, the transient activation of neutrophils observed after 15 minutes of dialysis may have resulted from the modest level of complement activation that is known to occur with both cellulose triacetate and polysulfone membranes [28, 29]. However, over the full course of the dialysis treatment, both membranes were associated with a normalization of the level of respiratory burst activity. We have previously shown that plasma from hemodialysis patients contains an unknown factor or factors capable of priming respiratory burst activity in normal neutrophils [18]. Other investigators, working with serum from hemodialysis patients [30] or peritoneal dialysis effluent [31, 32], have also shown the presence of a priming factor or factors with a molecular size less than 1.0 to 1.2 kD. The results of the present study are consistent with removal of this low-molecular-weight factor or factors, leading to a reduction in neutrophil priming.

The decrease in respiratory burst activity from pre- to post-dialysis was accompanied by changes in the markers of plasma protein oxidation. Plasma protein free sulfhydryl groups increased to normal, suggesting a reduction in oxidant stress. In contrast, protein carbonyl groups increased slightly in concentration, indicating a worsening oxidant damage. These apparently contradictory findings may be reconciled by considering the reactions involved in sulfhydryl group oxidation and carbonyl formation. Protein sulfhydryl groups can be oxidized through the formation of a disulfide bond with low-molecular-weight aminothiols, such as cysteine and homocysteine, present in plasma. This oxidation reaction is reversible. Cysteine is the most abundant aminothiol in plasma. Protein-bound cysteine concentrations are significantly increased in he-

Table 2. Concentrations of plasma protein oxidation markers in hemodialysis patients and normal subjects and the effect of high-flux hemodialysis

	Normal (N = 12 to 17)	F80A (N = 11)	CT190G (N = 11)
Free sulfhydryl groups *μmol/L*			
Pre-dialysis	438 ± 16	268 ± 22[a]	265 ± 19[a]
Post-dialysis		425 ± 15[b]	408 ± 23[b]
Carbonyl groups *nmol/mg*			
Pre-dialysis	0.041 ± 0.008	0.144 ± 0.037[a]	0.145 ± 0.030[a]
Post-dialysis		0.175 ± 0.029[ab]	0.178 ± 0.035[ab]
AOPP *μmol/L*			
Pre-dialysis	74 ± 8	191 ± 30[a]	191 ± 27[a]
Post-dialysis		190 ± 37[a]	163 ± 26[a]

Data are presented as mean ± SEM. [a] Different from normal; [b] Different from pre-dialysis

modialysis patients [33, 34] and decrease significantly from pre- to post-dialysis [34]. Similar findings have been reported for the less abundant aminothiol, homocysteine [33, 34]. In addition to aminothiols, protein sulfhydryl groups can be oxidized by reversible reactions with cyanate (CNO^-) [35], which spontaneously forms from urea under physiological conditions [36], and methylglyoxal [37], which is present in increased concentrations in dialysis patients [38]. Thus, removal by dialysis of free aminothiols, or other substances capable of binding reversibly to protein sulfhydryl groups, could lead to dissociation of their bound component from plasma proteins and restoration of the proteins' free sulfhydryl groups.

In contrast to the reversible nature of protein sulfhydryl group oxidation, the formation of protein carbonyl groups is irreversible [39]. Thus, the increased release of oxygen radicals early in dialysis could lead to an irreversible increase in protein carbonyl groups that was still evident, post-dialysis, despite an overall reduction in oxidant stress. High-flux dialysis had no effect on AOPP, suggesting that these substances are relatively large and that they also represent a state of irreversible oxidation. This conclusion is consistent with the studies of Witko-Sarsat et al [9], who found AOPP to result from irreversible oxidant-induced protein cross-linking and to be clustered at molecular weights of 70 kD and 670 kD. The exact nature of AOPP remains to be defined, however, and if they have significance beyond providing a global measure of protein oxidation has yet to be determined.

Protein oxidation may be a consequence of increased oxygen radical production, a deficiency in antioxidant systems, or both. Several studies, including the present one, have shown that oxygen radical production is increased in hemodialysis patients [18, 19, 30, 40, 41]. Superoxide dismutase, catalase, and the glutathione system provide major protection against damage by oxygen radicals. Studies of these enzymes in hemodialysis patients have yielded conflicting results. In erythrocytes, superoxide dismutase activity is reported to be normal or decreased [7, 42, 43], glutathione peroxidase activity to be normal [7, 42, 43], and glutathione reductase activity to be normal or decreased [7, 43]. Whole blood total glutathione concentrations are reported to be decreased [43], whereas erythrocyte total glutathione levels are reported to be normal [44]. However, superoxide dismutase, catalase, and the glutathione system are primarily intracellular antioxidants (for example, the plasma concentration of glutathione is only 2 μmol/L [45]) and they are unlikely to play a significant role in protecting against oxidation of plasma proteins. Important antioxidants in plasma include vitamins C and E, uric acid, and albumin [46]. Vitamin C levels have been reported to be low in some, but not all, hemodialysis patients [7, 47, 48]. We did not measure vitamin C levels in this study; however, five of the patients were taking a supplement containing vitamin C and it is unlikely that their vitamin C levels were markedly low. Vitamin E levels are reported to be normal in hemodialysis patients [7, 47]. Most free sulfhydryl groups in plasma are found on albumin [49] and a recent report by Himmelfarb and McMonagle showed that albumin is also a major target for protein carbonyl formation in plasma [50]. Taken together, these findings suggest that albumin may be an important defense against oxidant stress in hemodialysis patients. Such a role for albumin is supported by the finding of Soejima et al that patients with hypoalbuminemia demonstrate a greater degree of erythrocyte membrane lipid peroxidation than do patients with normal serum albumin concentrations [51]. It is intriguing to consider that a reduction in antioxidant capacity secondary to hypoalbuminemia might be one factor in the increased mortality associated with hypoalbuminemia in hemodialysis patients [52].

We [20], and others [30, 53], have shown previously that neutrophil oxygen radical production is increased in patients with renal insufficiency prior to the initiation of dialysis therapy. Such patients also exhibit evidence of plasma protein oxidation [6] and have elevated levels of AOPP [54]. Taken together, these observations suggest that uremia leads to a state of oxidant stress mediated by increased oxygen radical production. The results of our present study suggest that high-flux hemodialysis partially ameliorates this oxidant stress by removing solutes responsible for increasing neutrophil oxygen radical production and the low-molecular-weight aminothiols

involved in oxidation of protein sulfhydryl groups. From this perspective, high-flux hemodialysis could be considered protective against the oxidant stress of uremia rather than adding to oxidant stress as is often suggested.

The present study provides no information on whether low-flux hemodialysis also reduces oxidant stress. We reported previously that dialysis with low-flux cellulose membranes was associated with a reduction in neutrophil oxygen radical production from pre- to post-dialysis, albeit at a non-significant level [55]. Moreover, Himmelfarb, McMonagle, and McMenamin observed comparable increases in the concentration of protein free sulfhydryl groups from pre- to post-dialysis in patients treated with high-flux polysulfone membranes and low-flux cellulose membranes [6]. These observations are consistent with the view that oxidant stress involves a relatively low-molecular-weight agent (s) that is retained in uremia. Further studies comparing low-flux and high-flux hemodialysis and hemodiafiltration, which provides enhanced removal of higher-molecular-weight solutes, may be useful in more clearly defining the molecular weight of this retained solute (s). With dialysis three times a week, the amelioration of oxidant stress appears to be transient, since pre-dialysis neutrophil oxygen radical production and protein oxidation did not differ between the two dialyzers. Therefore, determining those dialysis strategies that best reduce this consequence of chronic uremia will also require studies of the time course of oxidant stress in hemodialysis patients. Friedman et al recently reported that plasma levels of the aminothiol, homocysteine, are significantly lower in patients treated with nocturnal hemodialysis six or seven nights per week compared to those in patients treated with standard three times a week dialysis [56], suggesting that frequency of treatment will be an important factor in reducing oxidant stress in hemodialysis patients.

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